

Ovalbumin-Induced IL-4, IL-5 and IFN- γ Production in Infants with Atopic Dermatitis

Mitsuaki Kimura Makiko Obi

Department of Allergy and Clinical Immunology, Shizuoka Children's Hospital, Shizuoka, Japan

Key Words

Hen's egg · Ovalbumin · Interleukin-4 · Interleukin-5 · Interferon-gamma · Cytokine · Atopic dermatitis · Lymphocyte proliferation

Abstract

Background: Although hen's eggs are considered a cause of infantile atopic dermatitis (AD), little is known about cytokine production upon egg stimulation in infants with AD. **Objective:** This study aimed to characterize the production of IL-4, IL-5 and IFN- γ upon stimulation with ovalbumin (OVA), a representative allergenic protein of egg, in infants with AD. **Methods:** Peripheral blood mononuclear cells (PBMCs) from 68 children with AD, including 46 infants (<1 year), were stimulated with OVA and the production of IL-4, IL-5 and IFN- γ was measured with ELISA kits. **Results:** Upon stimulation with OVA, the production of IL-4 and IL-5, but not IFN- γ , by PBMCs was significantly higher in infants with AD than in non-atopic controls. OVA-induced IL-5 production peaked in younger infants (2–5 months) and then decreased with age increase. In contrast, OVA-induced IL-4 production peaked at the age of 1–2. This coincided with the serum level of egg white-specific IgE (EW-IgE). There was a significant positive correlation between IL-5 production and the severity of symptoms in infants with AD, while IL-4 production significantly correlated with the serum level of EW-IgE. **Conclusion:** These results demonstrate that OVA-induced IL-5 production fluctuates with age in a dif-

ferent manner than IL-4 or EW-IgE. Our results suggest that egg contributes to the development of AD in younger infants by inducing the production of IL-5, but not IL-4.

Copyright © 2005 S. Karger AG, Basel

Introduction

Hypersensitivity to foods is assumed to precipitate the development of atopic dermatitis (AD) in early childhood [1–3]. The result of the elimination and challenge tests for foods demonstrates that hen's eggs are one of the most potent food allergens [4, 5]. Indeed, egg white-specific IgE antibody (EW-IgE) is positive in most infants with AD [6]. Because there is a positive correlation between the level of food-specific IgE antibody and the result of oral food challenge tests, food-specific IgE antibody appears to be involved in the development of skin lesions in children [7–9]. However, since oral food challenge tests ordinarily yields immediate hypersensitivity reactions of the skin such as hives but not AD due to allergic inflammation [4, 5, 7–9], it is still unclear whether food allergens actually induce AD.

Allergic inflammation is presently known to play a critical role in the development of AD [10]. Some investigators reported that lymphocyte proliferation and cytokine production upon stimulation with egg proteins is increased in children with AD [11, 12], or other allergic disorders [13, 14]. However, little is known about the

production of the representative proinflammatory cytokine IL-5 [15] upon egg stimulation in infants with AD. The production of IL-4, which enhances IgE synthesis [16, 17], upon egg stimulation is not clear in these subjects, either.

In this study, we examined the production of IL-4, IL-5, and IFN- γ by peripheral blood mononuclear cells (PBMCs) upon stimulation with ovalbumin (OVA) in children with AD. The results revealed that, although the production of both IL-4 and IL-5 was elevated in atopic subjects, the pattern of age-dependent variation as well as the correlation with symptoms or serum IgE levels is quite different between them.

Materials and Methods

Subjects

Heparinized peripheral blood was obtained at the time of routine blood examination from 68 children with AD who visited us between April 2001 and March 2004, after informed consent was obtained (table 1). Fifty-five of 68 subjects (80.9%) were positive for EW-IgE (>0.35 IU/ml). No subjects were given systemic corticosteroids. As controls, heparinized blood was similarly collected from 34 non-atopic children who did not have any serious diseases (table 1). This study was approved by the ethical review board of our institute.

AD was diagnosed by the criteria of Hanifin and Rajka [18]. The severity of AD was determined according to the criteria set by a task force of the Japanese Ministry of Health, Labor and Welfare as a part of a 2002 Guideline for the Treatment of Atopic Dermatitis. Briefly, mild: no severe skin lesions, moderate: severe skin lesions in less than 10% of the body surface, severe: severe skin lesions in between 10 and 30% of the body surface, and very severe: severe skin lesions in more than 30% of the body surface.

Cell Culture

Cell culture was performed according to the previously described method [19]. PBMCs were collected by centrifugation of blood samples over lymphocyte separation medium (ICN Pharmaceutical Inc., Tokyo, Japan) and resuspended at a concentration of 1×10^6 cells/ml in RPMI1640 medium (Sigma Aldrich Fine Chemical, Tokyo, Japan) supplemented with 10% heat-inactivated human AB serum, 2 mM glutamine and antibiotics (100 U/ml penicillin and 100 μ g/ml of streptomycin). The cell suspension was distributed in each well (0.5 ml/well) in duplicate on a 48-well culture plate (Falcon 3078, Becton Dickinson, N.J., USA). Cells were cultured for 6 days in a humidified atmosphere of 5% CO₂ at 37°C with or without 100 μ g/ml OVA. The concentration of OVA and the duration of culture were determined by preliminary experiments.

After incubation, the supernatant was collected and stored at -70°C until the measurement of cytokines. The cells were washed twice with PBS and fixed with 70% ethanol for the measurement of lymphocyte proliferation.

Table 1. Subjects

Age group	n	Age
AD		
2–5 months	17	4 (2–5) months
6–8 months	17	6 (6–8) months
9–11 months	12	9 (9–11) months
1–2 years	10	1 (1–2) years
3–15 years	12	5 (3–10) years
Controls		
0 years	13	5 (1–11) months
1–2 years	9	1 (1–2) years
3–15 years	12	5.5 (3–12) years

The number and age of the subjects in each age group are shown. Age is presented as the median and range.

Measurement of Cytokines and IgE Antibody

IL-4, IL-5, and IFN- γ in the culture supernatant was measured with the enzyme-linked immunosorbent assay (ELISA) kit (Cyto-screen human IL-4 (ultrasensitive), IL-5, and IFN- γ , BioSource International, Camarillo, Calif., USA), whose sensitivity was 200 fg/ml, 1 pg/ml, and 1 pg/ml, respectively. EW-IgE antibody was measured using a CAP-RAST RIA kit (Pharmacia KK, Tokyo, Japan).

Measurement of Allergen-Induced Lymphocyte Proliferation

Allergen-induced lymphocyte proliferation was measured as described previously [20]. Briefly, fixed cells were washed twice with PBS, and incubated in 500 μ l PBS containing 100 μ g/ml RNase (Sigma) at 37°C for 1 h. Cellular DNA was stained by 40 μ g/ml propidium iodide (Sigma). The amount of DNA per cell was analyzed by flow cytometry (EPICS Elite, Beckman-Coulter, Tokyo, Japan) and the S-phase cells that appeared in a flat region between the peaks of the G0/G1- and G2/M-phase cells were measured. The percentage of the S-phase cells was converted into a stimulation index using the following equation and expressed as SIF (stimulation index measured by flow cytometry).

$$\text{SIF} = (\text{S-phase cells (\%)} \text{ in a stimulated sample} / \text{S-phase cells (\%)} \text{ in an unstimulated sample}) \times 100$$

Statistics

All data are expressed as the median and range. The significance of differences was estimated by Mann-Whitney's U test and correlation was tested by Spearman's rank correlation coefficient. Results were regarded as significant if the p value was lower than 0.05.

Results

Variation of OVA-Induced IL-4 Production with Age Increase

IL-4 production upon OVA stimulation showed a marked age-dependent variation in children with AD (table 2). OVA-induced IL-4 production was low in younger

Table 2. Cytokine production

Age	LP (SIF)	Cytokine			EW-IgE UA/ml
		IL-4 fg/ml	IL-5 pg/ml	IFN- γ pg/ml	
AD					
2–5 months	186 ¹	<200	13.0 ²	<1	2.54
	88–1,333	<200–625	<1–287	<1–110	<0.35–>100
	17	16	14	13	17
6–8 months	163	<200	1.7	<1	9.08
	81–532	<200–740	<1–432	<1–50	<0.35–>100
	17	17	14	14	17
9–11 months	157	200	<1	<1	11.84
	75–552	<200–825	<1–28	<1–15	<0.35–>100
	12	12	11	11	12
1–2 years	188	484 ³	<1	1.2	58.28
	75–446	<100–1,550	<1–178	<1–46	<0.35–>100
	10	10	10	10	10
3–15 years	118	225	<1	<1	6.55
	77–181	<200–675	<1	<1–4.5	<0.35–96.3
	12	10	10	10	12
Controls					
2–11 months	114	<200	<1	<1	<0.35
	82–240	<200–547	<1–5	<1–7.5	<0.35
	13	11	9	11	13
1–2 years	130	<200	<1	3.2	<0.35
	94–244	<200–340	<1	<1–9	<0.35
	9	7	7	8	9
3–15 years	123	<200	<1	<1	<0.35
	96–156	<200–310	<1	<1–5	<0.35
	12	8	6	9	12

Lymphocyte proliferation (LP) upon OVA stimulation, cytokine production by PBMCs upon OVA stimulation and the serum level of egg white-specific IgE (EW-IgE) is shown as the median (top) and range (middle) along with the number of subjects (bottom).

SIF = Stimulation index measured by flow cytometry.

¹ Significantly higher than in control infants (2–11 months) ($p < 0.05$).

² Significantly higher than in control infants ($p < 0.02$) and 3- to 15-year-old children with AD ($p < 0.005$).

³ Significantly higher than in controls of 1–2 years ($p < 0.05$), infants with AD of 4–5 months ($p < 0.005$) and 6–8 months ($p < 0.05$).

infants with AD (2–5 months). Then it increased with age, peaked at the age of 1–2 years, and finally declined again in older children (3–15 years) (fig. 1). The production of IL-4 upon OVA stimulation in younger children with AD (1–2 years) was significantly higher than in non-atopic controls aged 1–2 years ($p < 0.05$), younger infants with AD (2–5 months) ($p < 0.005$) or intermediate-aged infants with AD (6–8 months) ($p < 0.05$).

Variation of OVA-Induced IL-5 Production with Age

OVA-induced IL-5 production also showed marked age-dependent variation (table 2). It peaked in younger infants with AD (2–5 months), and it was significantly

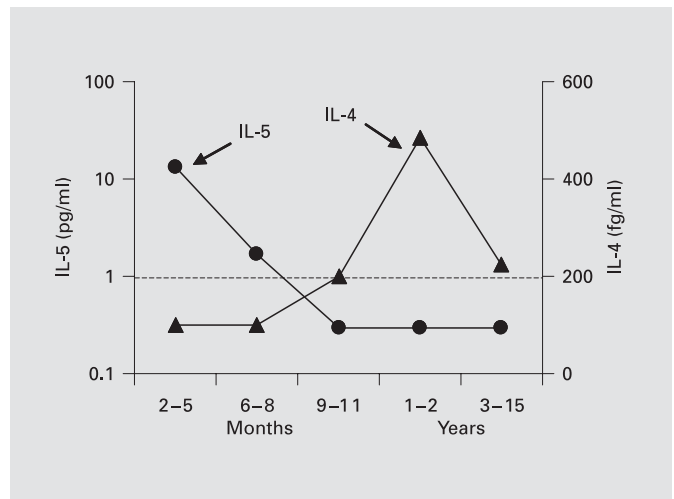
higher than in non-atopic infants (<1 year) ($p < 0.02$) or older children with AD (3–15 years) ($p < 0.005$). This pattern of variation of IL-5 production was quite different from that of IL-4 production (fig. 1).

Variation of OVA-Induced IFN- γ Production with Age

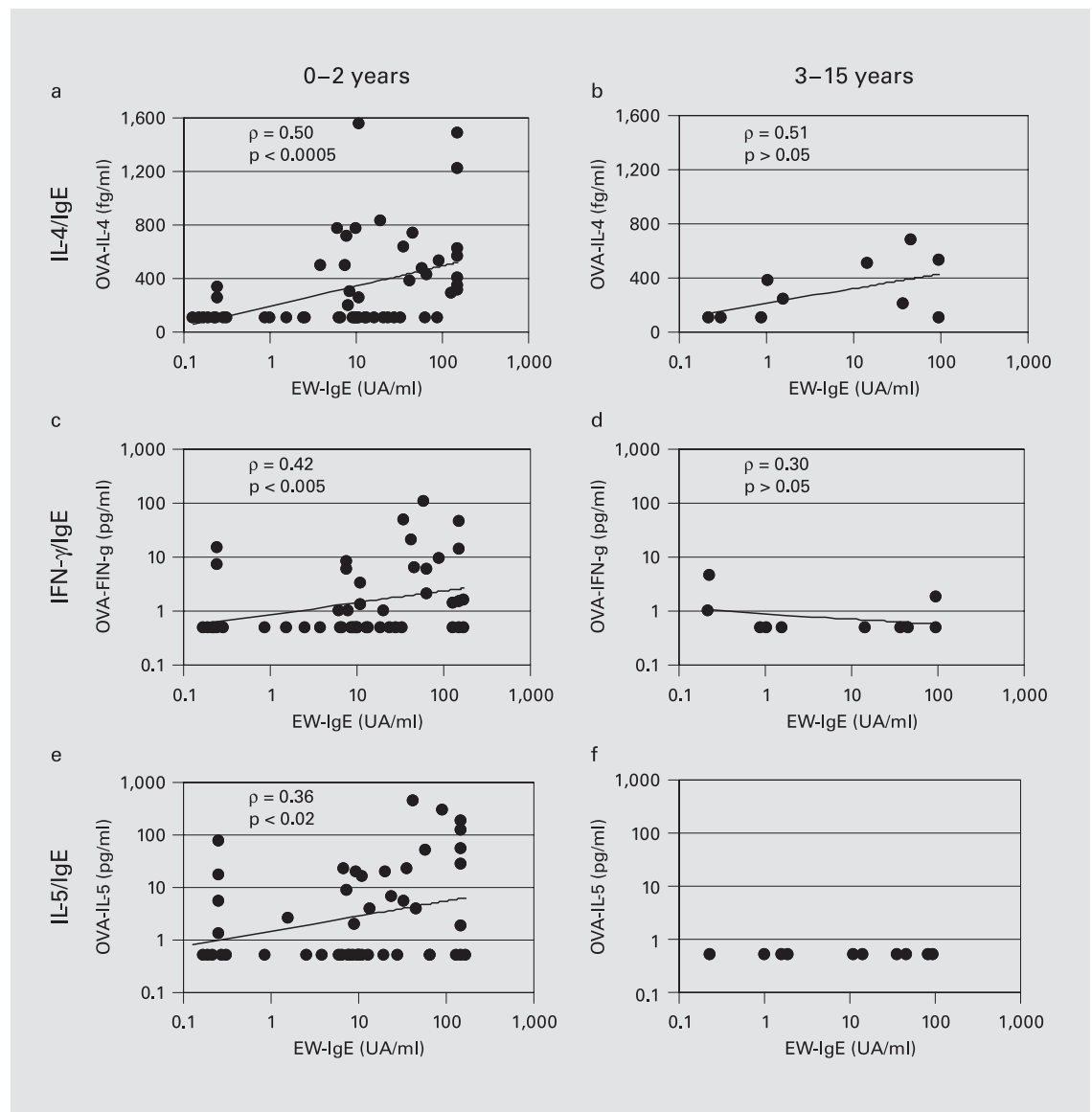
OVA-induced IFN- γ production in subjects with AD was not significantly different from that in control subjects (table 2). Although the mean IFN- γ production in younger children with AD (1–2 years) was lower than that in non-atopic counterparts (1.2 vs. 3.2 pg/ml), the difference was not significant.

Fig. 1. Variations with age in IL-4 and IL-5 production. The median of the production of IL-4 (triangle) and IL-5 (circle) upon OVA stimulation in each agegroup is shown. A dotted line in the figure indicates the lower limit of the measurable range.

Fig. 2. Correlation between cytokine production and serum EW-IgE level. Correlation of OVA-induced IL-4 (a, b), IFN- γ (c, d) and IL-5 (e, f) production with the serum level of egg white-specific IgE (EW-IgE) is shown. Subjects are limited to those with AD.



1



2

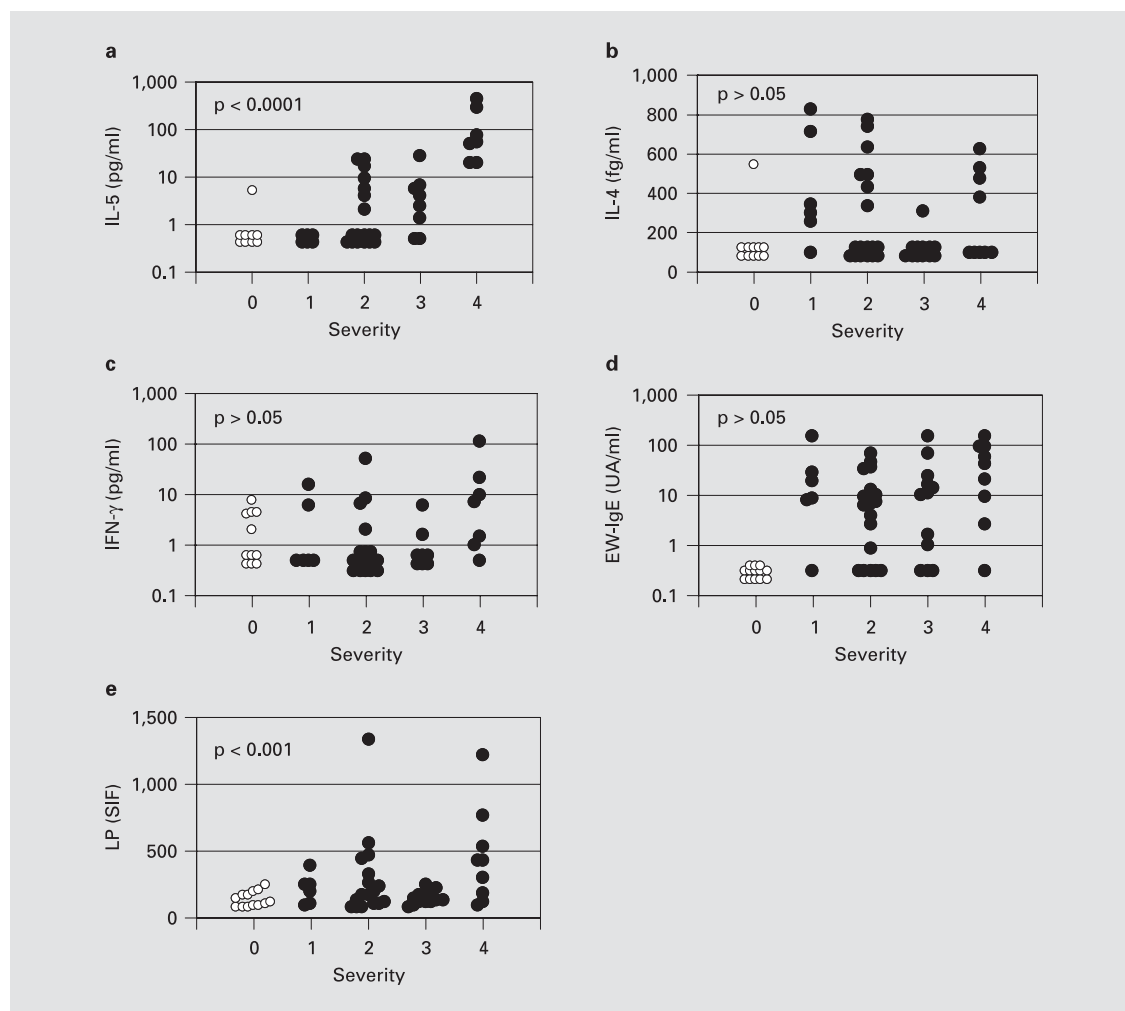


Fig. 3. Correlation of the severity of AD with cytokine production, EW-IgE and lymphocyte proliferation. Correlation of OVA-induced IL-5 production (**a**), IL-4 production (**b**), IFN- γ production (**c**), egg white-specific IgE levels (EW-IgE) (**d**) and OVA-induced lymphocyte proliferation (LP) (**e**) to the severity of symptoms of AD in infants (<1 year) is shown. Severity 0: control subjects; 1: mild; 2: moderate; 3: severe; 4: extremely severe. SIF = Stimulation index measured by flow cytometry.

There were no remarkable changes with age in OVA-induced IFN- γ production. Although a high production of IFN- γ (>10 pg/ml) was seen in some infants (<1 year) and younger children with AD (1–2 years), this high production was not seen in older children with AD (3–15 years).

Variation of OVA-Induced Lymphocyte Proliferation with Age

OVA-induced lymphocyte proliferation was significantly higher in younger infants with AD (2–5 months) than in control infants (<1 year) ($p < 0.05$) (table 2). This value decreased with age, and no differences were seen

between AD patients and control subjects after the age of 3 years.

Correlation of OVA-Induced Cytokine Production with EW-IgE

There was a significant positive correlation between OVA-induced IL-4 production and the level of serum EW-IgE (fig. 2a, $p < 0.0005$) in a combined group of infants and younger children with AD (0–2 years). OVA-induced IL-4 production was also positively correlated with the serum level of EW-IgE ($p = 0.50$) in older children with AD (3–15 years), although the correlation was not statistically significant (fig. 2b).

OVA-induced IFN- γ production showed a significant positive correlation with serum EW-IgE levels in a combined group of infants and younger children with AD (0–2 years) (fig. 2c, $p < 0.005$). In contrast, it was negatively correlated with serum EW-IgE levels in older children with AD (3–15 years) ($p = -0.30$).

The production of IL-5 upon OVA stimulation was also significantly correlated with the serum level of EW-IgE in a combined group of infants and younger children with AD (0–2 years) (fig. 2e, $p < 0.02$). However, lymphocyte proliferation upon OVA stimulation did not correlate with serum EW-IgE levels (data not shown).

Correlation of OVA-Induced IL-5 Production with the Severity of Symptoms

OVA-induced IL-5 production was significantly correlated with the severity of AD in infants (<1 year) (fig. 3a) ($p < 0.0001$). A significant positive correlation was also seen between lymphocyte proliferation upon OVA stimulation and the severity of symptoms in infants with AD (<1 year) (fig. 3e) ($p < 0.001$). The production of OVA-induced IL-4 (fig. 3b) or IFN- γ (fig. 3c), or serum levels of EW-IgE (fig. 3d) did not correlate with the severity of symptoms.

Discussion

IL-4 is a well-known Th2 cytokine that upregulates IgE synthesis [16, 17]. We previously showed that EW-induced IL-4 production is positively correlated with the serum level of EW-IgE, although the number of subjects in the study was limited [21]. In this study, we again demonstrated a positive correlation between OVA-induced IL-4 production and serum EW-IgE levels. Moreover, the variation of OVA-induced IL-4 production with age is consistent with that of serum EW-IgE levels. These findings suggest that IL-4 induced by OVA stimulation precipitates IgE synthesis for EW in atopic children.

Since IFN- γ has been shown to suppress IgE synthesis [16], it is expected to be unrelated or negatively correlated with the serum IgE level. Indeed, this is the case in IFN- γ production upon stimulation with HDM [21, 22] and *Candida albicans* [22]. However, in this study, it was found that OVA-induced IFN- γ production increased in proportion to serum EW-IgE levels in a combined group of infants and younger children with AD (0–2 years). The cause of such an apparently discrepant phenomenon remains to be elucidated. Although IL-4 appears to play an important role in the upregulation of IgE synthesis, the

function of IFN- γ should be reconsidered in those subjects with AD.

In AD patients, not only the serum level of EW-IgE and OVA-induced IL-4 production but also OVA-induced IFN- γ production is highest in younger children (1–2 years). Among those factors, only IFN- γ production is lower compared with non-atopic counterparts. This may suggest that, although IFN- γ production apparently increases in proportion to serum EW-IgE levels, it is insufficient to substantially suppress IL-4-induced IgE synthesis.

IL-5 is another representative Th2 cytokine that facilitates allergic inflammation by promoting the growth and activation of eosinophils [10, 15]. Eosinophils and their products such as ECP are thought to play a critical role in the pathogenesis of AD [23, 24]. We previously reported that IL-5 production by PBMCs upon stimulation with HDM or Japanese cedar pollens (JCP) was already increased in infants with AD, despite a lack of IgE for these allergens in the serum [20, 25]. In the present study, we also demonstrated that stimulation of PBMCs with OVA induces a large amount of IL-5 production in infants (<1 year) with moderate to severe AD. Food allergens have been regarded as a cause of AD in infants [1–3]. Although EW-IgE is usually positive in those subjects, it alone cannot explain the development of AD due to allergic inflammation. This study indicates that IL-5 may be a possible mediator by which egg induces AD in infants.

IL-5 production, as well as lymphocyte proliferation was highest in younger infants with AD (2–5 months). This is in sharp contrast to EW-IgE, which begins to rise during the first year of life and peaks at or after 1 year of age [6]. However, this result corresponds to the fact that AD in children usually develops during the first 6 months of life [1]. We also observed that AD develops before the age of 5 months in more than 90% of cases of infantile AD [unpubl. observation]. Thus, this observation again suggests that egg proteins, including OVA, contribute to the development of AD in infants by inducing IL-5 production.

Egg is seldom ingested by infants as food before the age of 5 months since weaning usually starts thereafter. This means AD develops in infants before oral ingestion of egg. It is interesting to consider how infants with AD come into contact with egg proteins before weaning. A possible way of transfer of egg proteins to infants at the time of the development of AD is breast milk. Indeed, breast milk has been demonstrated to contain various food proteins, including OVA, ingested by mothers [26].

Thus, breast milk may be responsible for the development of AD in infants before weaning.

It is notable that the incidence of elevated IL-5 production by OVA-stimulated PBMCs is rapidly lowered with age, suggesting that the potential of EW to cause skin lesion due to allergic inflammation decreases in children older than 1 year. This may correspond to the finding that clinical symptoms induced by oral food challenge tests for egg, which are ordinarily applied to children after the age of 1, are usually those belong to immediate hypersensitivity reactions due to IgE [4, 5].

A rapid increase and a following drastic decline associated with age increase in IL-5 production is not unique to OVA. Similar changes correlating to age are seen in JCP-induced IL-5 production, which also peaks in infants (<1 year) [25]. This pattern clearly differs than that of HDM-induced IL-5 production, which remains high even after the age of 1, similar to IL-4 production [13, 27]. Such variations cannot be detected by the use of polyclonal mitogens [28]. The recognition of these variations may be essential for a proper understanding of the role of each allergen in the development of allergic disorders.

References

- Sly RM: Atopic dermatitis; in Behrman RE, Kliegman RM, Jenson HB (eds): Nelson Textbook of Pediatrics, ed 16. Philadelphia, Saunders, 2000, pp 680–684.
- Halken S, Høst A, Hansen LG, Østerballe O: Effect of an allergy prevention programme on incidence of atopic symptoms in infancy. *Allergy* 1992;47:545–553.
- Hide DW, Matthews S, Tariq S, Arshad SH: Allergen avoidance in infancy and allergy at 4 years of age. *Allergy* 1996;51:89–93.
- Sampson HA: Role of immediate food hypersensitivity in the pathogenesis of atopic dermatitis. *J Allergy Clin Immunol* 1983;71:473–480.
- Burks AW, Mallory SB, Williams LW, Shirrell MA: Atopic dermatitis: Clinical relevance of food hypersensitivity reactions. *J Pediatr* 1988; 113:447–451.
- Rowntree S, Cogswell JJ, Platts-Mills TAE, Mitchell EB: Development of IgE and IgG antibodies to food and inhalant allergens in children at risk of allergic disease. *Arch Dis Child* 1985;60:727–735.
- Sampson HA, Ho DG: Relationship between food-specific IgE concentrations and the risk of positive food challenges in children and adolescents. *J Allergy Clin Immunol* 1997;100: 444–454.
- Sicherer SH, Morrow EH, Sampson HA: Dose-response in double-blind, placebo-controlled oral food challenges in children with atopic dermatitis. *J Allergy Clin Immunol* 2000;105: 582–586.
- García-Ara C, Boyano-Martínez T, Díaz-Pena JM, Martín-Muñoz F, Reche-Frutos M, Martín-Esteban M: Specific IgE levels in the diagnosis of immediate hypersensitivity to cow's milk protein in the infant. *J Allergy Clin Immunol* 2001;107:185–190.
- Leung DYM: Atopic dermatitis: New insights and opportunities for therapeutic intervention. *J Allergy Clin Immunol* 2000;105:860–876.
- Kondo N, Agata H, Fukutomi O, Motoyoshi F, Orii T: Lymphocyte responses to food antigens in patients with atopic dermatitis who are sensitive to foods. *J Allergy Clin Immunol* 1990;86:253–260.
- Kondo N, Fukutomi O, Agata H, Motoyoshi F, Shinoda S, Kobayashi Y, Kuwabara N, Kameyama T, Orii T: The role of T lymphocytes in patients with food-sensitive atopic dermatitis. *J Allergy Clin Immunol* 1993;91:658–668.
- Ng TW, Holt PG, Prescott SL: Cellular immune responses to ovalbumin and house dust mite in egg-allergic children. *Allergy* 2002;57: 207–214.
- Smart JM, Kemp AS: Increased Th1 and Th2 allergen-induced cytokine responses in children with atopic disease. *Clin Exp Allergy* 2002;32:796–802.
- Romagnani S: The role of lymphocytes in allergic disease. *J Allergy Clin Immunol* 2000; 105:399–408.
- Coffman RL, Carty J: A T cell activity that enhances polyclonal IgE production and its inhibition by IFN- γ . *J Immunol* 1986;136:949–954.
- Pene J, Rousset F, Briere F, Chretien I, Bonnefoy JY, Spits H, et al: IgE production by normal human B cells is induced by interleukin-4 and suppressed by interferon- α and β and prostaglandin E₂. *Proc Natl Acad Sci USA* 1988; 85:6880–6884.
- Hanifin JM, Rajka G: Diagnostic features of atopic dermatitis. *Acta Dermatovener* 1980; 92(suppl):44–47.
- Kimura M, Obi M, Saito M: Japanese cedar pollen-specific interleukin-4 production develops immediately after the first exposure to pollens in infants with atopic dermatitis. *Clin Exp Allergy* 2004;34:1032–1036.
- Kimura M, Tsuruta S, Yoshida T: Correlation of house dust mite-specific lymphocyte proliferation with interleukin 5 production, eosinophilia, and the severity of symptoms in infants with atopic dermatitis. *J Allergy Clin Immunol* 1998;101:84–89.
- Kimura M, Tsuruta S, Yoshida T: Unique profile of IL-4 and IFN- γ production by peripheral blood mononuclear cells in infants with atopic dermatitis. *J Allergy Clin Immunol* 1998;102:238–244.
- Kimura M, Tsuruta S, Yoshida T: IFN- γ plays a dominant role in upregulation of Candida-specific IgE synthesis in patients with atopic dermatitis. *Int Arch Allergy Immunol* 2000; 122:195–199.
- Kiehl P, Falkenberg K, Vogelbruch M, Kapp A: Tissue eosinophilia in acute and chronic atopic dermatitis: A morphometric approach using quantitative image analysis of immunostaining. *Br J Dermatol* 2001;145:720–729.
- Czech W, Krutmann J, Schopf E, Kapp A: Serum eosinophil cationic protein (ECP) is a sensitive measure for disease activity in atopic dermatitis. *Br J Dermatol* 1992;126:351–355.
- Kimura M, Obi M, Saito M: Japanese cedar pollen-specific IL-5 production in infants with atopic dermatitis. *Int Arch Allergy Immunol* 2004;135:343–347.
- Kilshaw PJ, Cant AJ: The passage of maternal dietary proteins into human breast milk. *Int Arch Allergy Immunol* 1984;75:8–15.
- Kimura M, Yamaide A, Tsuruta S, Yoshida T: Development of the capacity of peripheral blood mononuclear cells to produce IL-4, IL-5 and IFN- γ upon stimulation with house dust mite in children with atopic dermatitis. *Int Arch Allergy Immunol* 2002;127:191–197.
- Neaville WA, Tisler C, Bhattacharya A, Anklam K, Gilbertson-White S, Hamilton R, Adler K, Dasilva DF, Roberg KA, et al: Developmental cytokine response profiles and the clinical and immunologic expression of atopy during the first year of life. *J Allergy Clin Immunol* 2003;112:740–746.